Additional Tests on the Efficacy of Ginger Root Oil in Enhancing the Mating Competitiveness of Sterile Males of the Mediterranean Fruit Fly, *Ceratitis capitata* (Diptera: Tephritidae)

Todd E. Shelly[1], Ernie Steiner[2], Vanessa Bosco[3], and **Donald McInnis[4]**
[1]USDA-APHIS, 41-650 Ahiki Street, Waimanalo, HI 96795, and Center for Conservation Research and Training, University of Hawaii, Honolulu, HI 96822.
[2]Department of Agriculture, 3 Baron Hay Court, South Perth, WA, 6151, Australia.
[3]Entomology Unit, South Australian Research and Development Institute, GPO Box 397, Adelaide, 5001, SA, Australia. [4]USDA-ARS, 2727 Woodlawn Drive, Honolulu, HI 96822.

Abstract. Recent studies have shown that exposure to the aroma of ginger root oil (*Zingiber officinale* Roscoe; termed GRO hereafter) increases the mating competitiveness of males of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). This result suggests that pre-release exposure of sterile males to GRO might increase the effectiveness of the Sterile Insect Technique (SIT) against this important agricultural pest. Here, we present the results of two experiments that further investigate the utility of GRO in medfly SIT. In the first, we compare the effectiveness of GRO obtained from three different suppliers in enhancing the mating success of sterile males relative to wild-like males in competition for wild-like females. Following adult exposure to GRO in large, holding boxes, we found significant variation in the mating success of sterile males exposed to GRO from different sources. However, regardless of the source, GRO-exposed males obtained significantly more matings than control, non-exposed males. In the second experiment, we found that the introduction of GRO (using two different doses) to closed, paper buckets at the time of pupal placement resulted in increased mating success of subsequently emerged sterile males. The use of GRO in SIT programs is discussed in light of these findings.

Introduction

Recent studies (Shelly et al. 2004a and references therein) have shown that exposure to ginger root oil (*Zingiber officinale* Roscoe; termed GRO hereafter) increases the mating competitiveness of males of the Mediterranean fruit fly (or medfly), *Ceratitis capitata* (Wiedemann). These studies have generated several observations suggesting that pre-release, adult exposure to GRO, which contains a powerful male attractant (the hydrocarbon sesquiterpene, α-copaene; Flath et al. 1994), may be useful in improving the effectiveness of the Sterile Insect Technique (SIT) against this pest: 1) Males need not feed on GRO to gain a mating advantage; exposure to the aroma alone is effective (Shelly 2001); 2) GRO is effective at low doses. For example, application of as little as 0.125 ml of GRO to the screen-covered top of a large eclosion box (a so-called PARC box, holding ≈ 36,000 males in 0.1 m^3 volume) increased mating success approximately 50% above that observed for control males deprived of GRO (Shelly et al. 2004a); 3) Because of the high potency, GRO exposure is a relatively inexpensive procedure. For example, at a dose of 0.5 ml per PARC box, GRO exposure would cost approximately $1.20 per million males or less than 1% of the cost of sterile flies for the California Preventative Release Program ($133 per million; E. Y. Smith, personal communication); 4) GRO exposure confers heightened mating success for as long as 8-10 d after exposure (Shelly 2001); 5) Wild females initially mated to

wild males are more likely to remate with GRO-exposed sterile males than with GRO-deprived sterile males (Shelly et al. 2004b); and 6) Exposure to GRO does not negatively affect male survivorship (Shelly et al. 2004a) or flight propensity (J. Zermano, personal communication).

The present study describes results from two additional experiments on the effect of GRO on the mating success of sterile male Mediterranean fruit flies (medflies). Both of these projects were stimulated by queries from the Western Australian SIT project on medfly, which is currently considering incorporation of GRO exposure in their program. First, we examined whether GRO obtained from three suppliers is equally effective in boosting male mating success following adult exposure. All previous studies (Shelly et al. 2004a and references therein) were conducted using GRO from a single supplier in the USA, and hence the present study assesses the robustness of the procedure by using GRO from different, international sources.

Also, smaller SIT programs (e.g., Western Australia) may use enclosed paper buckets, rather than PARC boxes, to hold eclosed males. In these instances, food and pupae are introduced simultaneously, and the buckets remain closed until the adult males are released (3–5 d later). Consequently, the second objective of our study was to determine whether GRO was effective when placed in buckets along with the pupae. Because pupae are placed in holding containers 1-2 d before eclosion and GRO is effective on adults only, it is possible that pre-emergence volatilization may reduce the impact of GRO on male sexual behavior. Shelly et al. (2004a) obtained mixed results when GRO was added to the PARC boxes at the time of pupal placement: male mating was enhanced at a dose of 1 ml of GRO but not at a dose of 0.5 ml. Because 1) PARC boxes and paper buckets differ greatly in size (see below) and 2) addition of GRO at the time of pupal placement may reduce processing time, we considered it worthwhile to re-test the efficacy of this technique with the paper buckets.

Materials and Methods

Study animals. Mass-reared males were from a *tsl* strain (Vienna-7/Tol-99) produced by the California Department of Food and Agriculture Hawaii Fruit Fly Rearing Facility, Waimanalo, Oahu, HI. The strain has been mass-reared at the USDA-APHIS facility in El Pino, Guatemala, since 1999, and in 2001 ≈ 1.25 million eggs from this facility were used to start the colony in Hawaii. Like other *tsl* strains, Vienna-7/Tol-99 possesses a sex-linked mutation, such that treating the eggs with high temperature kills all female zygotes, thereby allowing production of male exclusively (Franz et al. 1996). Males used in the current study were irradiated as pupae 2 d before eclosion in air at 150 Gy of gamma irradiation from a ^{137}Cs source.

Because wild flies were not available in sufficiently large numbers, we used flies from a recently established colony in the mating trials. These flies, hereafter termed "laboratory" males and females, were derived from a colony started with 400–500 adults reared from coffee (*Coffea arabica* L.) collected on the island of Kauai. Adults were held in screen cages and provided with a sugar-protein (yeast hydrolysate) mixture (3:1 by weight), water, and an oviposition substrate (perforated plastic vials containing small sponges soaked in lemon juice). Eggs were placed on standard larval diet in plastic containers over vermiculite for pupation. Adults used in the mating trials were separated by sex within 24 h of eclosion, well before reaching sexual maturity at 5–7 d of age. Laboratory flies (as well as *tsl* males) were held at 23–27 °C at 60–90% RH under natural and artificial light with a photoperiod of 12:12 h (L:D). When used in the current study, laboratory flies were 4–5 generations removed from the wild.

Experiment 1: variable performance among different GRO. After irradiation, *tsl* pupae were placed in paper bags (100 ml of pupae per bag; 1 ml contains ≈ 60 pupae), and six bags were placed in individual PARC boxes. Most males emerged 2 d after pupal placement, and emerging *tsl* males were fed a sugar-agar gel placed on the screened opening on the top of the box. GRO was applied to PARC boxes for a 24-h period starting 3 d after the day of peak adult emergence. At 0800 hrs, we placed 0.5 ml of a given GRO (see below) on blotter paper (5 cm square) resting on the screen-covered top of the PARC boxes. For a given test day, we placed GRO on three PARC boxes (one for each of the three types of GRO tested, see below), while another box received no GRO at all and served as the control. The four PARC boxes were each kept in separate rooms to avoid olfactory "contamination" among the different treatments. The laboratory males were not exposed to any GRO in any of the mating trials.

Immediately after exposure, we removed one paper bag (and the males resting on it) from each storage box, quickly transferred it to a screen cage, and gently shook it to disperse the males. Males were then marked for mating tests to be conducted the following day (i.e., when *tsl* males were 5 d old). For a given trial, we marked either the laboratory (7–11 d old) or the *tsl* males, alternating the marked group between successive trials. Males were cooled for several minutes, and a dot of enamel paint was placed on the thorax. This procedure had no obvious adverse effects, and males resumed normal activities within minutes of handling. After marking, males were held in screen-covered, plastic buckets (5 liters volume) with ample food (sugar-agar gel for *tsl* males and the sugar-protein mixture for laboratory males).

We investigated GRO from three different chemical suppliers (Table 1; for brevity, the oils are identified using the nationality of the supplier). The companies procured the oil from India or China and then processed it to meet governmental and company specifications. Extraction methods varied, but the physical and chemical properties of the different GRO were similar. The concentration of α–copaene was 0.4% in the Australian and American GRO but exceeded 2% in the Chinese GRO. The cost of the American GRO was approximately 20% lower than that of either the Australian or Chinese GRO.

Experiment 2: GRO exposure in paper buckets. The objective of this experiment was to determine whether placing GRO-impregnated, filter paper discs (8 mm diameter) in paper buckets simultaneously with *tsl* pupae resulted in increased mating competitiveness of the subsequently eclosed *tsl* males. We placed 60 ml of *tsl* pupae (the same volume used in the Australian medfly SIT program) in a Petri dish on the bottom of the paper buckets (5 liters volume). At the same time, we also placed two Petri dishes containing sugar agar gel and sugar-protein mixture, respectively, in the buckets. For buckets containing treated *tsl* males, we applied either 0.0625 ml or 0.1 ml of GRO (from the American supplier) to a paper disc (resting in a small plastic dish) using a micropipette and placed this on the bottom of the bucket. Control *tsl* males were handled in the same manner but were not exposed to GRO. Buckets containing treated and control *tsl* males were held in separate rooms to avoid inadvertent exposure to the control males. Males were aspirated from the paper buckets 1 d prior to testing when they were marked (following the above procedure) and held in plastic buckets with food and water. When tested, control and treated *tsl* males were 5 d old. Laboratory flies were handled following the above procedure. As in the first experiment, laboratory males were not exposed to any GRO in any mating trial.

Mating trials. Mating trials were conducted at the USDA-ARS-PBARC facility, Honolulu, HI, between April-June, 2004. Groups of 75 laboratory females, 75 laboratory males, and either 75 treated (exposed to GRO) *tsl* males or 75 control (no GRO exposure) *tsl* males were released between 0800-0830 hrs in field cages (height 2.5 m; diameter 3.0 m) that contained two artificial trees (each 2 m tall with ≈ 450 leaves resembling those of *Ficus*

Table 1. Characteristics of GRO used in Experiment 1. The different GROs are identified by the country in which the supplier is located.

	Supplier nationality		
Parameter	Australia	China	USA
Supplier	Australian Botanical Products, Hallam, Victoria	Novanat Biological Co., Qingdao	Citrus & Allied Essences Ltd., Lake Success, NY
Country of origin	India	China	China
Extraction method	Steam	Carbon dioxide	Steam
Appearance	Pale amber to greenish	Light yellow	Pale yellow
Specific gravity	0.872–0.895[1]	0.873–0.888[2]	0.870–0.882[2]
Optical rotation	-25 to -55[1]	-45 to -56[2]	-28 to -47[NA]
Refractive index	1.480–1.498[1]	1.482–1.503[1]	1.488–1.494[1]
% alpha-copaene	0.4%	2.2%	0.4%
Cost (USD/kg)	$101	$102	$78

[NA] Not available; [1] at 20° C; [2] at 25° C

benjamina L.). Artificial trees were used, because the available host trees (e.g., common guava, *Psidium guajava* L., and orange, *Citrus sinensis* L.) contain chemicals (α-copaene among them) that affect the sexual behavior of male medflies (Shelly et al. 2004c; Shelly and Villalobos 2004). The artificial trees provided a chemically neutral substrate on which the flies displayed the entire complement of natural activities. The cages were monitored for 3 h, mating pairs were collected in vials, and the males identified.

For experiment 1, mating trials were conducted on 14 different days, and on each day we ran four trials (in four adjacent tents), representing the three different treatments (i.e., three GROs) and the associated control. For experiment 2, mating trials were conducted on five different days for each dose, and on each day we ran four trials, representing two replicates each for treated and control *tsl* males. During the trials, air temperature ranged from 25-30 °C.

Statistical analyses. As the assumptions of normality and equal variance were always met, the Students t test and ANOVA were used to make pair wise and multiple comparisons, respectively. Proportions were arcsine transformed in these tests. Following detection of significant variation in ANOVA, the Tukey multiple comparisons test was used to identify significant differences between groups. Means (± 1 SD) are presented. Analyses were performed using SigmaStat Statistical Software (Version 2.0).

Table 2. Mating success of treated and control *tsl* males competing against laboratory males for copulations with laboratory females. Treated *tsl* males were exposed to GRO from suppliers in the USA, Australia, or China; control *tsl* and laboratory males were not exposed to GRO. Means (± 1 SD) are given; n = 14 in all cases.

	Number of matings				% total matings
Supplier	*tsl* males	Laboratory males	t	P	*tsl* males
USA	29.5 (7.4)	26.8 (6.3)	1.0	0.32	52.1 (8.0)
Australia	26.8 (6.8)	30.8 (7.1)	1.5	0.15	46.5 (8.7)
China	23.4 (4.4)	33.4 (7.7)	4.2	< 0.001	41.4 (7.6)
None (control)	9.2 (6.3)	34.1 (9.8)	8.0	< 0.001	20.8 (11.3)

Results

Experiment 1: variable performance among different GRO. Pair wise comparisons showed that treated *tsl* males exposed to GRO from suppliers in the USA or Australia achieved similar number of matings as laboratory males (Table 2). In contrast, laboratory males obtained a significantly greater number of matings than treated *tsl* males exposed to Chinese GRO or control *tsl* males (Table 2).

Because there was significant variation in the total number of matings observed among treatments ($F_{3, 52} = 7.1$; $P < 0.001$; ANOVA), we made between-treatment comparisons using relative mating success (% total matings). Significant variation was detected among *tsl* treatment categories in relative mating success ($F_{3, 52} = 29.6$; $P < 0.001$; ANOVA). A subsequent multiple comparisons test (Tukey's test; $P = 0.05$) revealed that treated *tsl* males accounted for a higher proportion of the total matings than control *tsl* males regardless of the source of the GRO. On average, treated *tsl* males obtained 41%-52% of all matings compared to only 21% for control males (Table 2). Thus, while both control *tsl* males and treated *tsl* males exposed to Chinese GRO were competitively inferior to laboratory males (as noted above), exposure to Chinese GRO nonetheless increased the proportion of matings obtained by *tsl* males. Only a single difference was detected among the treated *tsl* males: treated *tsl* males exposed to GRO from the USA supplier accounted for a significantly higher proportion of matings than treated *tsl* males exposed to GRO from China ($P < 0.05$; Tukey test). No difference in relative mating success was detected between *tsl* males exposed to the Australian and Chinese GRO, respectively (Tukey test, $P > 0.05$).

Experiment 2: GRO exposure in paper buckets. For both doses tested, placing GRO-laden paper in paper buckets at the time of pupal placement increased the mating success of *tsl* males. At the 60 µl dose, treated *tsl* males obtained 20.4 (10.1) matings per replicate compared to 29.0 (8.8) matings for laboratory males ($t = 1.8$; $P = 0.09$; $df = 18$). In contrast, control *tsl* males achieved only 9.2 (5.6) matings compared to 43.3 (7.5) matings for laboratory males ($t = 11.5$; $P < 0.001$; $df = 18$). On average, treated *tsl* males obtained a significantly higher proportion of matings than control *tsl* males (41% versus 17%, respectively; $t = 3.1$; $P = 0.01$; $df = 18$).

At the 100 µl dose, treated *tsl* males obtained 16.6 (6.5) matings per replicate compared to 25.4 (7.2) matings for laboratory males ($t = 2.9$; $P = 0.01$; $df = 18$), whereas control *tsl* males achieved only 7.7 (3.4) matings compared to 33.6 (7.0) matings for laboratory males ($t = 11.0$; $P < 0.001$; $df = 18$). On average, treated *tsl* males obtained a significantly higher proportion of matings than control *tsl* males (39% versus 19%, respectively; $t = 5.4$; $P = <$

0.001; df = 18). The proportion of matings obtained by treated *tsl* males did not differ significantly between the 60 and 100 μl doses (t = 0.11; P = 0.91; df = 18).

Discussion

The present study has produced three noteworthy results. First, regardless of the source, exposure of *tsl* males to GRO increased the mating competitiveness significantly above that observed for non-exposed, control males. Control *tsl* males obtained an average of only 21% of the total matings per replicate compared to 41%-52% for treated *tsl* males exposed to one of the GROs tested. Although the mechanism is still unknown, the GRO-mediated increase in male mating success is consistent with results from other mass-reared strains as well wild populations of the medfly (Shelly et al. 2004a and references therein).

Second, although GRO-exposed males, in general, had a mating advantage over control males, their performance relative to wild males varied with the source of the GRO. Following exposure to GRO from Australia or the USA, *tsl* males obtained a similar number of matings per replicate as laboratory males. In contrast, laboratory males achieved significantly more matings per replicate than *tsl* males exposed to GRO from China. The relative mating success of *tsl* males exposed to American GRO (mean = 52%) was significantly greater than that observed for *tsl* males exposed to Chinese GRO (mean = 41%), while the difference in relative mating success was not significantly different between *tsl* males exposed to Australian (mean = 46.5%) and Chinese GRO, respectively. The fact that the concentration of α-copaene was greatest in the GRO that produced the smallest increase in male mating success (i.e., the Chinese GRO) indicates: 1) there is an optimal level of α-copaene in GRO that maximizes improvement in male mating performance and that this optimal level is not necessarily the maximum and/or 2) other compounds, including possibly other sesquiterpenes, may affect the sexual behavior of male medflies alone or jointly with α-copaene, and the concentration of these other compounds may vary among the different GRO. Based on the present findings, it appears that GRO from the US supplier is best suited for application to medfly SIT, because 1) it yielded the highest relative mating success among treated *tsl* males, and 2) it had the lowest cost per kg.

Third, data from the paper buckets revealed that placing the GRO simultaneously with the pupae resulted in increased male mating success. As GRO is known to be effective on adults only (Shelly 2001), the odor retained within the closed buckets was apparently sufficient to impact male mating behavior.

In conclusion, the present study provides further evidence of the robustness of GRO as a tool in medfly SIT. Although differences existed among the three suppliers, exposure to any of the GRO tested boosted male mating success over that observed for control males. Thus, availability would not appear to be an impediment to the implementation of GRO in medfly SIT programs. In addition, GRO has proven effective under widely varying conditions of male eclosion, from paper buckets (5 liters volume) holding approximately 3,600 males (this study) to PARC boxes (0.1 m^3 volume) holding about 36,000 males (Shelly et al. 2004a) to entire trailers (175 m^3 volume) holding about 13 million males (Shelly, unpublished data). Thus, the method of holding eclosed males does not appear to restrict application of the GRO procedure, although testing may be required to identify the effective dose for particular holding environments.

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